REMARKS

Claims 1, 2, 6, 7, 10, and 39-43 are pending in the application. Claims 1, 2, 6, 10, and 39-43 are rejected. The specification has been amended to reflect the current status of nonprovisional patent applications referenced in the first line of the specification.

Double Patenting

Claims 1, 2, 6, 7, 10 and 39-43 are rejected for double patenting over USSN 10/430,176. Applicants respectfully refer the Examiner to M.P.E.P. § 822.01 which states that such double patenting rejections are provisional until such time as the provisional double patenting rejections are the only rejections remaining in the applications. At that time, "the examiner should then withdraw that rejection in one of the applications and permit the application to issue as a patent" (M.P.E.P. § 822.01). Applicants therefore acknowledge that the double patenting rejections are provisional and do not require response until such time that the rejections are converted into double patenting rejections (M.P.E.P. § 822.01).

Claim Rejections - 35 U.S.C. § 112

Claims 1, 2, 6, 7, 10 and 39-43 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. In particular, the Examiner asserts that specification provides written description support for (Applicants assume that by "for conception", the Examiner is referring to written description support) Claim 1 and claims dependent thereon for "administering [in] an effective dose of an anti-coagulation factor IX/IXa monoclonal antibody having self-limiting neutralizing activity". Applicants have amended Claim 1 to limit the claim to a method wherein the monoclonal antibody has self-limiting neutralizing activity. As this is in compliance with the Examiner's affirmative statement regarding written description, Applicants respectfully request withdrawal of the rejection.

The Examiner also asserts that the specification does not provide written description support for Claim 41 because the subgenus recited in the claim (binding to an epitope of the Factor IX gla domain) "lacks conception as filed in this application". The Examiner supports this assertion by referring to "M.P.E.P. § 2163.05(b)" and by stating that the recited limitation does not appear in the specification and therefore introduces

"new concepts". Applicants traverse and state that the specification provides adequate support for this subgeneric claim. As an initial matter, Applicants are unable to locate in the M.P.E.P. a subsection (b) of section 2163.05, and requests that the Examiner provide clarification. Moreover, Applicants respectfully assert that the Examiner has arbitrarily chosen to rely on a single decided case cited in the M.P.E.P. while ignoring the other cited cases. Section II of M.P.E.P. § 2163.05 clearly cites case law contrary to the Examiner's position (see *In re Lukach* and *Ex parte Sorenson*), and instructs that the issue raised by the Examiner is to be decided on its own unique facts "in terms of what is reasonably communicated to those skilled in the art" (citing *In re Wilder*). The instant facts are closer to those considered by the Board in *Ex parte Sorenson*, a decision that acknowledges the holding in the case cited by the Examiner (*In re Smith*) but held that the "originally filed disclosure reasonably convey[ed] to the skilled artisan that [the inventor] had possession of that subject matter" (*Ex parte Sorenson*, 3 USPQ2d1462, 1464 (BPAI 1987)). The Examiner recites the Smith case as if it laid down a *per se* rule. However, the M.P.E.P. makes it clear that "a change involving subgeneric terminology may or may not be acceptable". Finally, the M.P.E.P. explicitly states that all claim limitations need not be expressly supported in the originally filed disclosure but may be implicitly or inherently supported (see M.P.E.P. § 2163.05, first paragraph). Applicants respectfully assert that localization of the precise binding site of one or more of the anti-coagulation factor IX/IXa monoclonal antibodies to an epitope or epitopes within the gla domain would lead on skilled in art to appreciate that they were in possession of the invention as claimed in Claim 41.

The Examiner also rejects Claim 42, stating that "there is no conception antibody epitopes that is located within residues 3-11 of Factor IX". Applicants have amended Claim 42 to clarify that the monoclonal antibody binds to an epitope located within residues 3-11 of the Factor IX gla domain.

Finally, the Examiner rejects Claim 43 for recitation of the limitation "wherein the antibody has a binding affinity of at least 4nM", and request Applicants to point out in the instant specification support for that limitation. Applicants direct the Examiner's attention to Table 1 on page 57, and particularly lines 17-18 wherein it is indicated that the Factor IX Binding Affinity is 4 nM.

Claims 1, 2, 6, 7, 10 and 39-43 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the

subject matter which Applicants regards as the invention. The Examiner alleges that it is unclear whether the antibody binds to Factor IX, Factor IXa, or both. Applicants respectfully assert that it is clear from the instant specification that the monoclonal antibodies that are useful in the claimed methods bind to Factor IX and/or Factor IXa. See page 6, line 19 to page 7, line 5. See also page 14, line 1 to page 15, line 5, Table 1 at page 38, and Table 4 at page 60.

Claim 1 is also rejected as indefinite for recitation of "an effective dose": the Examiner states that "it is not clear from the claim construction what effect is achieved by the amount". Applicants traverse and respectfully assert that the claim is clear on its face: the effect achieved is inhibition of thrombosis in an animal.

Regarding Claim 6 and 7, Applicants traverse and respectfully assert that in view of the wealth of data provided in the specification, not the least of which are the nucleic and amino acid sequences of the CDRs of the recited monoclonal antibodies, as well as binding kinetics, affinity, etc., one skilled in this art would have no trouble appreciating the identifying characteristics of the claimed monoclonal antibodies.

Claim 41 is rejected as indefinite allegedly because Applicants have failed "to set forth epitopes of the Factor IX gla domain". Applicants respectfully traverse and assert that the gla domain of Factor IX was well known and appreciated by those skilled in this art at the time the invention was made. See, for example, the Cheung et al. reference cited in the instant specification at page 59, line 17, a copy of which is provide herewith for the Examiner's convenience.

Finally, Claim 42 is rejected as indefinite due to "recitation of specific residues [of the gla domain] in the absence of the corresponding sequence". Applicants respectfully traverse and assert that gla domain of Factor IX was well known to those skilled in the art at the time the invention was made. See the Cheung et al. reference provided herewith, and the Cheung et al. reference cited in Applicants' IDS. Accordingly, the claim is not indefinite.

Claim Rejections - 35 U.S.C. § 102 and 103

Claims 1, 6, 7, 10, 39 and 40 are rejected under 35 U.S.C. 102(e) as being clearly anticipated by Griffin et al., U.S. Patent No. 5,679,639. Applicants traverse and respectfully assert that the instant claims, as amended herein, are not anticipated by the Griffin et al. disclosure. The instant claims are drawn to methods for inhibiting

thrombosis using monoclonal antibodies specific for Factor IX/IXa and that exhibit self-limiting neutralizing activity. Griffin et al. does not disclose or suggest antibodies having self-limiting neutralizing activity and therefore cannot anticipate the instant claims.

Claims 41-43 are rejected under 35 U.S.C. § 103(a) over Griffin et al., U.S. Patent No. 5,679,639 in view of Cheung et al. Applicants traverse and assert that Cheung et al. does not repair the infirmity of the Griffin et al. reference. Neither reference discloses or suggests the used on anti-coagulation factor IX/IXa monoclonal antibodies having self-limiting neutralizing activity.

In view of the foregoing amendments and remarks, Applicants respectfully submit that the subject application is in condition for allowance. If the Examiner has any remaining objections or concerns, the Examiner is respectfully requested to contact Applicants' undersigned attorney to resolve such issues and advance the case to issue.

Respectfully submitted,

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LOCALIZATION OF A METAL-DEPENDENT EPITOPE TO THE AMINO TERMINAL RESIDUES 33-40 OF HUMAN FACTOR IX

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Abstract Metal binding sites within the Gla domain of vitamin K-dependent coagulation factors have been divided into nonspecific metal sites and calcium-specific sites. We demonstrate here that five residues within the Gla domain of factor IX are responsible for the reactivity with the metal-dependent factor IX monoclonal antibody, A-7. First we demonstrate that modifying any one of three residues within this site in factor IX abolishes the binding of A-7. To confirm the specificity of the antibody, the Gla domain of factor VII was changed at residues 32, 33, 34, 38 and 39 to the homologous residues of human factor IX. These changes were sufficient to generate a factor VII Gla domain with an A-7 binding site of the same affinity as that in factor IX. The site identified is one of the two major surfaces of the Gla domain and may represent the metal-dependent binding site.

The amino-terminal glutamic acid residues of the vitamin K-dependent plasma proteins are modified to γ-carboxyglutamic acid (Gla) (1, 2). The Gla domain, containing these modified amino acids, is highly homologous among the vitamin K-dependent proteins. Although the Gla domains can bind various divalent metal ions including calcium, magnesium, strontium, and manganese, only calcium and strontium support reaction with a phospholipid surface and physiological activity (3). The crystal structure of bovine prothrombin fragment 1 (4) shows seven bound calcium ions forming a complex network at the N-terminus of the Gla domain. Furthermore, it was found that eight strontium ions

Key words: Factor IX, metal-dependent antibody, epitope mapping

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can bind to prothrombin fragment 1. The structure of Sr-prothrombin fragment 1 is similar to that of Ca-fragment 1 with some significant differences. The extra Sr^{2+} ion is suggested to be involved in metal ion-phospholipid interactions, along with other Sr^{2+} ions (5).

Based on a number of chemical and biochemical studies by intrinsic fluorescence quenching (6, 7), circular dichroism (8), and equilibrium binding (9), metal ion binding sites within the Gla domain have been classified into two major types. Various divalent metal ions can fill three high-affinity, cooperative, cation-nonspecific sites, while calcium ions are needed to fill several lower-affinity, cation-specific binding sites (9). Borowski *et.al.* (10) investigated these metal ion binding requirements with conformation-specific antibodies to the prothrombin Gla domain. They concluded that metal ion binding to the Gla domain causes a two-state transition. The first transition occurs when metal ions bind at non-cation-specific sites, and the second transition occurs when calcium or strontium binds to cation-specific sites, producing the phospholipid binding structure. In a similar study with polyclonal antibodies to the factor IX Gla domain, Liebman *et.al.* (11) demonstrated distinct metal and calcium binding states within this domain. They showed that when factor IX binds a number of different divalent cations, including manganese, magnesium, calcium, strontium and barium, it expresses a conformational antigenic determinant recognized by metal-dependent antibodies. Moreover, when factor IX binds calcium or strontium, a second conformational antigenic determinant is expressed which is recognized by calcium-dependent antibodies. Fab fragments of the metal-nonspecific antibodies affected neither the activation of factor IX by factor XIa nor the binding of factor IX molecules to phospholipid vesicles, while Fab fragments of the calcium-specific antibodies inhibited both interactions. They concluded that the two antibody populations bound to distinct regions of the factor IX Gla domain and that the two regions were dependent on the formation of specific metal ion-binding states (11). However, neither the cation specificities of these binding sites nor the physiological significance of each of the binding sites is well understood.

Sekiya *et.al.* (12) showed that in the presence of calcium, the binding of factor IX to the snake venom anticoagulant, IX/X-bp, to calcium-specific conformational polyclonal antibodies, and to calcium-dependent anti-Gla domain monoclonal antibodies is greatly augmented by Mg^{2+} ions at physiological concentrations, as is the activation of factor IX by factor XIa. These results suggest the presence of a Mg^{2+}-specific binding site that does not interact with Ca^{2+} ions in factor IX. Our studies here define the regions of the factor IX Gla domain required for binding to a monoclonal antibody, A-7 (13), whose characteristics appear similar to the metal-dependent polyclonal antibodies previously described (11).

MATERIALS AND METHODS

Dulbecco's modified Eagles medium (DMEM), fetal bovine serum and Geneticin (G418) were from GIBCO/BRL (Gaithersburg, MD). Vitamin K (Aquamephyton) was obtained from Merck Sharp and Dohme. Restriction and modifying enzymes were from United States Biochemical Corp. (Cleveland, OH). Anti-human factor IX monoclonal antibodies, A-1, A-5 and A-7 have been described previously (14, 15). Human embryonal kidney

cell line 293 (16) was from ATCC (Rockville, MD). Factor IX was purified from plasma as previously described (13). Iodobeads were from Pierce (Rockford, IL).

Site-directed mutagenesis, production of recombinant proteins and measurement of reactivity with monoclonal antibody A-7.
Site-directed mutagenesis was made according to the method of Stanssens *et.al.* (17), on cDNAs of human factor VII, factor IX and a chimera, which has factor VII Gla and aromatic domains connected to the epidermal growth factor-like and catalytic domains of factor IX. Mutated cDNAs were ligated into the mammalian expression vector pCMV5 (18). Mutants were expressed in human 293 cells grown in DMEM containing 10% fetal bovine serum and 10 μg/ml vitamin K. Supernatants were analyzed for expression with monoclonal antibodies A-5 (anti-residues 180-310) and A-1 (anti-residues 147-153) of factor IX (19). Removable wells (Immulon 1, Dynatech Laboratories, Chantilly, VA) were coated with antibody A-5 at 10 μg/ml in TBS (0.05 M Tris-HCl, 0.1 M NaCl, 0.005 M $CaCl_2$, 0.001 M $MgCl_2$, pH 7.5) overnight and were then blocked with 3% ovalbumin. The wells were washed three times with TBS, and tissue culture supernatants were then added and incubated for 2 hr at 37°C. After the wells had been washed three times with TBS, the reactivity with A-1 or A-7 was assessed by adding 100,000 cpm of radiolabeled antibody per well and incubating at room temperature for 2 hr. The wells were then washed five times with TBS and counted in a gamma counter. The percent reactivity of mutated proteins with A-7 were normalized with A-1 (anti-activation peptide MoAb).

Competition for A-7 binding.
^{125}I-factor IX was labeled with Iodobeads to a specific activity of 3 μCi/μg; its concentration was determined by the Bio-Rad Protein Assay Kit. Removable wells were coated overnight with 0.1 ml A-7 antibody at 10 μg/ml in TBS at 4°C and then blocked with 3% ovalbumin in TBS at 4°C. The wells were washed three times with TBS, followed by addition of 0.09 ml of unlabeled factor IX or mutated factor VII at concentrations of 0.1 to 100 nM in TBS with 1% ovalbumin. Ten microliters of 10 nM of radiolabeled factor IX were added to the wells and mixed with competing antigen. After incubation at room temperature for 2.5 hr, the wells were washed five times and counted in a gamma counter.

RESULTS

Smith *et.al.* (13) showed that monoclonal antibody A-7 is a metal-dependent antibody, since either calcium or other divalent metal ions can induce binding to factor IX. The calcium concentration for half-maximal binding of A-7 to factor IX was 3.5 mM, and a similar magnesium ion concentration was required (13).

Amino acid substitutions and antibody binding.
Because we knew that bovine factor IX does not interact with the anti-human factor IX MoAb, A-7, we initially made point mutations of human factor IX at Met 19 and Arg 37, the two residues which differ between the bovine and human proteins in the Gla domain. It was found that replacing Arg 37 with Lys (the amino acid in bovine factor IX) abolished

A-7 reactivity; however, the substitution of Met 19 by Lys from the bovine sequence did not affect A-7 binding (Fig. 1). Additional factor IX point mutations which destroyed A-7 reactivity were at positions 36 and 40. None of the Gla domain mutations listed (Fig. 1) affected the recognition of factor IX by the monoclonal antibodies, A-1 or A-5, which bind to the activation peptide and heavy chain of human factor IX, respectively (19). Moreover, calcium specific antibodies JK.IX1-4 (20) bound to the mutated molecules with no apparent decrease in affinity (data not shown). Also, multiple mutations in the first 11 amino acids had no effect on A-7 binding (Fig. 1).

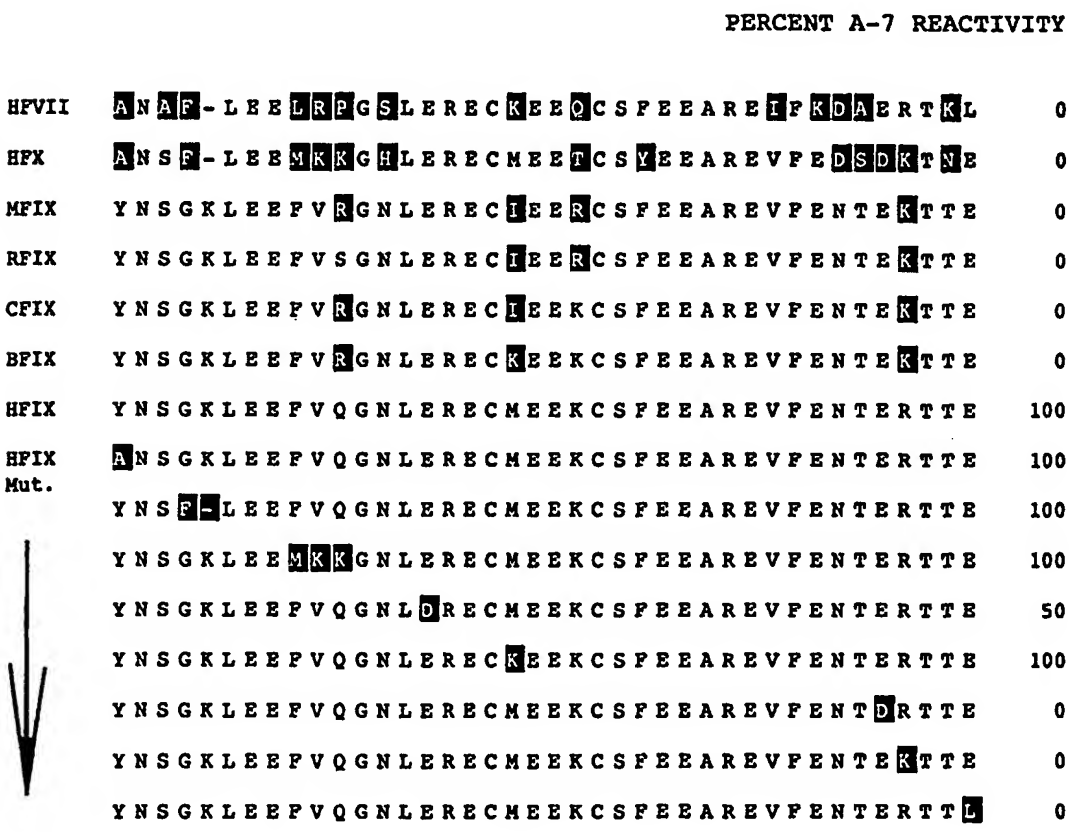

FIG. 1.

The reactivity of monoclonal antibody A-7 with factor IX and its mutants. Residues different from those of wild-type human factor IX are shown in black. Only the Gla domain is shown. HFVII, human factor VII; HFX, human factor X; MFIX, mouse factor IX; RFIX, rabbit factor IX; BFIX, bovine factor IX; HFIX, human factor IX, and HFIX mut., human factor IX mutants. The reactivity with A-7 is shown as percentage relative to that of recombinant and plasma factor IX.

It is possible that mutations of Glu residues 36 or 40 (Fig. 1) may have affected the formation of the metal-induced conformation. However, this is less likely, as the Arg residue at position 37 is also very critical for A-7 reaction; replacement of this residue with Lys, without changing any Glu residues, thus completely eliminated A-7 binding (Fig. 1). Moreover, Gla analysis of several of the purified recombinant proteins showed an average of 9-10 Gla residues per molecule, indicating that the mammalian expression system is capable of producing normal carboxylated proteins (21).

As eliminating an epitope is less convincing than creating an epitope, we attempted to create an A-7 epitope in the Gla domain of factor VII and a factor VII / IX chimera. The Gla domain of factor VII contains ten Gla residues, while factor IX has twelve such residues, the additional Gla residues being located in positions 33 and 40. We first introduced Glu residues at either or both of the homologous positions (32 and 39) in the Gla domain of factor VII (Fig. 2, construct 2, 3, and 4, respectively; construct 4 thus contains all twelve glutamic acid residues of factor IX). None of these constructs reacted with the metal-dependent monoclonal antibody, A-7. However, it was found that changing five residues of the factor VII Gla domain to those of factor IX (K32E, D33N, A34T, K38T and L39E; construct 5) was sufficient to create a binding site for A-7 with the same Kd, ~16 nM, as that of purified factor IX (Fig. 3). Factor VII and chimeric factor VII/IX molecules with these five residues changed are efficiently purified by A-7 affinity chromatography (21, 22). Furthermore, the chimeric factor VII/IX protein with these five residues mutated can be activated by factor XIa (21, 22).

Construction			PERCENT A-7 REACTIVITY
hFVII	1	ANAF-LEELRPGSLERECKEEQCSFEEAREIFKDAERTKLFW	0
	2	ANAF-LEELRPGSLERECKEEQCSFEEAREIFEDAERTKLFW	0
	3	ANAF-LEELRPGSLERECKEEQCSFEEAREIFKDAERTTEFW	0
	4	ANAF-LEELRPGSLERECKEEQCSFEEAREIFEDAERTTEFW	0
	5	ANAF-LEELRPGSLERECKEEQCSFEEAREIFENTERTTEFW	100
hFIX	6	YNSGKLEEFVQGNLERECMEEKCSFEEAREVFENTERTTEFW	100

FIG.2.

The reactivity of monoclonal antibody A-7 with Gla region mutants of factor VII. Residues in the Gla region of factor VII changed to the corresponding residues of factor IX are shown in black. HFVII, human factor VII; HFIX, human factor IX. The reactivity with A-7 is given as percentage relative to that of recombinant and plasma factor IX.

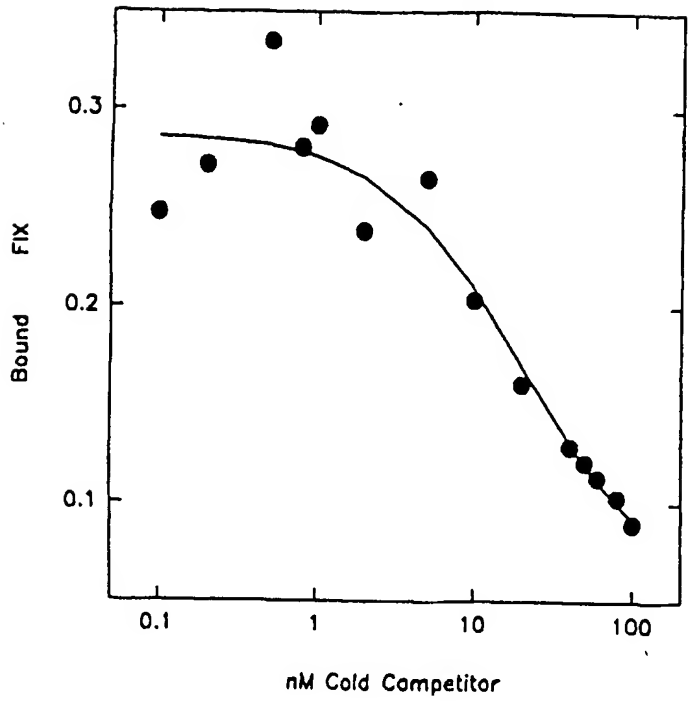

FIG. 3

Competition between mutated factor VII (construct 5) and plasma factor IX for A-7 binding. Wells were coated with monoclonal antibody A-7, and were incubated with ^{125}I-factor IX (final concentration 1 nM) together with increasing concentrations of unlabeled mutated factor VII.

DISCUSSION

Differences in the conformations of vitamin K-dependent coagulation factors in the presence of magnesium and calcium ions were originally described in a series of publications from Furie's laboratory (10, 11, 23-25). They suggested that conformational changes progress from a metal ion-dependent state (conformation I) to a calcium ion-specific state (conformation II). The functional importance of these two states has not been demonstrated, but the two classes of binding sites have been confirmed by Borowski *et.al.* (10) and Liebman *et.al.* (11). The high specificity required for calcium binding can be attributed to structure of the Gla domain. The seven calcium ions observed in the crystal structure of the Gla domain of prothrombin (4) are very tightly packed, so that only calcium can fit. In the case of the metal-dependent site, the structure is much looser and almost any divalent cation will suffice to give the correct structure.

We recently published (21) a model of the factor IX Gla domain which predicted two major accessible surface areas, residues 1-11 (the omega loop), and residues 33-40. It is likely that these two areas mediate specific physiological functions, although these functions are not yet fully defined. In this work, we have characterized a metal ion-dependent epitope, which includes residues 33-40, and possibly other residues common between factor IX and factor VII, and which is recognized by antibody A-7. Unpublished results with calcium-dependent monoclonal antibodies JK.IX1-4 (20) and conformation-specific polyclonal antibodies (Wojcik, E. and Bertina, R. M., personal communication) suggest that the calcium-specific sites are in the amino-terminal portion of factor IX.

Monoclonal antibody A-7 inhibits activation of factor IX by factor XIa, indicating that the A-7 epitope is located close to the site of recognition by factor XIa. In contrast, phospholipid does not affect the binding between factor IX and A-7 in a solid phase assay, consistent with previously published results from other laboratories, that the amino-terminal region of the Gla domain is involved in phospholipid binding. For instance, mutation of Leu 5 to Gln in protein C was shown to severely impair the binding of the mutant protein to phospholipid vesicles (26). Furthermore, the solution structure and computer-modelled structure of the Gla domain of bovine factor X suggested that the hydrophobic residues Phe 4, Leu 5 and Val 8 are exposed to the solvent and are inserted into the phospholipid membrane (27). Moreover, point mutations of Lys 5 in factor IX to Ala and Arg, or of Val 10 to Lys, resulted in normal *in vitro* clotting activity (21) and approximately normal interaction with phospholipid (Wolberg, A.S., unpublished results). However, multiple mutations of residues 4, 5, 9, 10, or 11 in factor IX gave reduced *in vitro* clotting activity, although these constructs could be activated by factor XIa. The results implicated that this region involved in the interaction with phospholipid membranes, as well as the platelet surface (21, 22).

Additional evidence that the binding site of the monoclonal antibody A-7 is characteristic of metal-dependent sites is suggested by independent studies. Antisera raised against a peptide (residues 28-46), which includes part of the Gla domain and the aromatic stack region of factor IX, reacts with an epitope which is destroyed by the addition of either calcium or magnesium ions (28). Thus, both calcium and magnesium cause a conformational change in this region of factor IX. The ability of an antibody to recognize the particular conformation (with or without cation) would depend on the stability of the conformation and the fit of the cation in the three-dimensional structure of this region.

The localization of the metal ion-dependent antibody binding determinants (residues 33-40) to a surface-exposed region of the Gla domain will be useful in studies of the function of this region in coagulation and may help clarify data on metal ion binding by vitamin K-dependent coagulation factors in general.

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